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The effect of EDTA and Heparin anticoagulants on the survival of Neutrophils and T-cell responses among Visceral Leishmaniasis Patients in North West Ethiopia

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Acronyms and Abbreviations

APCs	Antigen-presenting cells
CD	Cluster of differentiation
CL	Cutaneous Leishmaniasis
Co_2	Carbondioxide
DCL	Diffuse Cutaneous Leishmaniasis
DCs	Dendritic Cells
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FITC	Fluorescein Isothiocyanate
GAGs	Glycosaminoglycans
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay
IL	Interlukin
LCL	Localized Cutaneous Leishmaniasis
LDGs	Low Density Granulocytes
MCL	Muco-cutaneous Leishmaniasis
NDGs	Normal Density Granulocytes
NET	Neutrophil Extracellular Traps
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohemaglutinin
PHOS	Phagocytosis associated oxidase
PM	Paramomysin
rK- 39	Recombinant Kinesin 39 amino acid
ROS	Reactive oxygen species
SLA	Soluble Leishmania Antigen
SOP	Standard Operating Procedures
SSG	Sodium stibogluconate
TOC	Test of cure
VL	Visceral Leishmaniasis
WBA	Whole Blood Assay

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Abstract

Background: The leishmaniasis are a group of diseases caused by protozoan parasites of the genus *Leishmania*. *Leishmania* are, obligate intracellular kinetoplastid protozoan parasites, that are transmitted through the bite of a vector called sandy fly. The disease ranges from self-healing cutaneous to visceral leishmaniasis (VL), which is severe and can causes death when untreated. Following the delivery of parasites by sand fly bite or by needle injection, neutrophils have been found the first recruited to the infection site. The role of neutrophils in either promoting or suppressing host immune response remains controversial.

Objective: To assess the effect of EDTA and heparin anticoagulants on the survival of LDGs in PBMCs and the release of interferon-γ and IL-10 in the WBA.

Methods: A cross-sectional study was conducted on clinically and laboratory confirmed VL patients from December 2014 to May 2015 at University of Gondar Hospital. Convenience sampling technique was used to select study participants and all consecutive VL cases who came to the hospital during the study period and controls were included. 6 ml of blood using EDTA and 6 ml using heparin was collected from all study participants before and after treatment and from controls to assess the effect on LDGs using FACs caliber, arginase activity using Enzymatic assay and production of IFN-γ and IL-10 using ELISA. Data were evaluated using GraphPad Prism 6 and differences were considered statistically significant at $p < 0.05$. FACS data were analyzed using Summit v4.3 software.

Results: The frequency of LDGs is significantly lower when the blood is collected with heparin as compared to EDTA. Anticoagulants does not have an impact on the levels of arginase released. And results show that EDTA prevents the production of IFN-γ. Cells from active VL patents produce no or low levels of IFN-γ and IL-10, however, after successful treatment, these cells gradually regain their capacity to produce IFN-γ, but not IL-10.

Conclusion and recommendations: In this study results suggest that, VL patients have lost their ability to mount a Th1 response during active VL and that active disease is not associated with sustained levels of IL-10. For the future in vitro experiment, choosing different anticoagulants to study about different cells and T cell response against leishmania is important.

Key Words:- *Leishmania, Neutrophil, Heparin, EDTA, IFN - gamma*

1. Introduction

1.1 Background

The leishmaniasis are a group of diseases caused by protozoan parasites of the genus *Leishmania*. *Leishmania* are, obligate intracellular kinetoplastid protozoan parasites that are transmitted through the bite of a vector called sandy fly. The disease ranges from self-healing cutaneous to visceral leishmaniasis (VL), which is severe and can causes death when untreated (1).

Globally, endemic leishmaniasis was reported in a total of 98 countries. The reported cases of cutaneous leishmaniasis per year was more than 220,000 (2).

Visceral leishmaniasis is fatal disease if left untreated. Estimated annual global incidence of VL is 200,000–400,000 and more than 90% of cases occur in countries like Sudan, India, Bangladesh, South Sudan, Brazil and Ethiopia (2).

In Ethiopia, VL is caused by *L. donovani* and it is one of the most significant vector-borne diseases; Ethiopia has the second largest number of VL cases in sub-Saharan Africa with an estimated annual burden of 4500 to 5000 new cases (3).

There are different forms of cutaneous leishmaniasis. Such as Localized cutaneous leishmaniasis (LCL), Mucocutaneous Leishmaniasis (MCL) and Disseminated cutaneous leishmaniasis (DCL) LCL heals spontaneously but it can take months to years to heal and varies depending on the species (4). MCL is a type of leishmaniasis which is chronic, caused by *L. braziliensis, L. panamensis,, L. guyanensis and L. amazonensis,* typically seen in central and South America. and may spread to mucosal membrane, particularly to the throat, mouth and nose and can cause extensive damage that leads to disfiguration (5).

Visceral leishmaniasis or kala-azar is usually caused by *L. donovani* and *L. infantum*, and the disease is characterized by splenomegaly, weight loss, progressive fever, hypergammaglobulinemia, pancytopenia and hepatomegaly (6) and VL also causes different complications like immunosuppression and secondary bacterial infections, hemorrhage and anemia (7).

Because *leishmania* are obligate intracellular parasites, the humoral immune response is not effective and cell mediated immunity is required for control of infections caused by all *leishmania* species (8).

In experimental model, immune responses that are mediated by T-cell play a central role in the processes of either cure or aggravation of the disease. Resistance to *Leishmania* infection depends on polarization of the immune response towards T-helper-1 type which induces production of IL-12 and IFN-γ, while disease progression is related to development of T-heper-2 type of immune response in which IL-4 and IL-5 have a significant contribution (9).
Following the delivery of parasites by sand fly bite or by needle injection, neutrophils have been found the first recruited to the infection site (10).
The best-characterized function of neutrophils or polymorphonuclear neutrophils is their role in the phagocytosis and killing of invading microorganisms via the generation of oxygen intermediates and the lytic enzymes released from their granule (11).

Neutrophils also takes part during inflammation process which is an important step to combat pathogens at early phase of infection. Furthermore, an immune regulatory role of neutrophil has been identified via the secretion of cytokines and chemokines. This role is additional to their being key component of inflammatory response (12), Neutrophils are shown to contribute to the recruitment and activation of antigen-presenting cells (APCs) (13). Thus, neutrophils are now recognized as important decision shapers during the early phases of the immune response (11).

Neutrophils are short-lived leukocytes that die by apoptosis, necrosis, and Neutrophil Extracellular Traps (NETosis). However, following infection, their lifespan can be increased to several days (14).

Upon death by NETosis, neutrophils release fibrous traps of deoxyribosenucleic acid (DNA), histones, and granule proteins named NETs, which can kill bacteria and fungi. Studies done with *leismania amazonensis* show the release of NETs by human neutrophils upon their interaction with *leishmania* parasites. *Leishmania* and the lipophosphoglycan expressed on the parasite surface induced NET release by neutrophils, important for killing of the parasite (15).

Based on the expression of CD28 surface markers Polymorphoneuclear neutrophils can be divided into either CD28+ or CD28- cells on the basis of their capacity to express CD28. CD28+ and CD28- neutrophils phagocytose *Leishmania* parasites and secret Interleukin-8 that amplifies the neutrophils at the site of infection (16).
About 47% human peripheral blood neutrophils express CD28 and that CD28 signals through IP3 kinase will induces IFN-γ and T cell chemotactic factor, suggesting the role of neutrophils in initiation of T cell response against *leishmania* (17).

Leishmania parasites exist in two distinct morphological forms in mammalian host promastigote and Amastigote. The motile flagellated promastigote exist, multiply and develop extera cellularly in the alimentary tract of the female sand fly vector (18). During the blood meal Promastigotes are phagocytosed by macrophages or neutrophils (19). In vacuolar compartments of macrophage Promastigotes will differentiate to another stage called amastigote. Amastigotes multiply by binary cell division and can spread to other phagocytic cells like dendritic cells (DC) and other macrophages (20).

Given that, leismaniasis is a main public health problem, finding new and effective control measures are commendable. Control of leishmaniasis usually depends on case detection, treatment, vector and reservoir control. Early detection of the disease and treatment are essential for individual patients and also for the community. If VL patients are treated in an early stage, the outcome of the treatment will be better. VL patients that are not treated can act as a source of infection and then contribute to disease transmission (21,22).

1.2 Statement of the problem

Neutrophils are readily mobilized innate immune cells and are essential for host defense mechanism against different pathogens (23). Evidence is emerging that PMN can play a part in antigen presentation, and as a result this could have an impact on the induction and the quality of the T-cell response (24).

The role of neutrophils in either promoting or suppressing host immune response remains controversial. Data indicates that PMN can also have immunosuppressive functions. Polymorhoneuclar neutrophils are having different effector functions during pathogen invasion. The first is through L-arginine metabolizing enzymes, arginase and nitric oxide synthase (NOS), and the second is using phagocytosis-associated oxidases (PHOX) that produce reactive oxygen species (ROS). Both ROS (25) and nitrogen intermediates (26) have the ability to inhibit T cell activation.

Arginase is the enzyme that hydrolyzes arginine and catalyzes the hydrolysis of L-arginine to urea and ornithine. Arginase I and II are the two isoforms of the enzyme arginase. Arginase I is a trimeric cytosolic protein and is expressed in erythrocytes in humans and higher primates. Arginase II, which is the second isoform, is also a trimeric mitochondrial protein, and is expressed in extrahepatic tissues like brain, kidney, small intestine, macrophages and monocytes (27). Arginase II is synthesized as a pre-protein, and processed to the mature form after imported to mitochondria (28).

Efficient activation of T cells is depending on the availability of L-arginine and has been shown to be inhibited when extracellular L-arginine is depleted (26,29). Human PMN constitutively express high amounts of arginase I, which is contained within their azurophilic granules (30). Arginase release from PMN either by active degranulation or by their death leads to depletion of extracellular L-arginine, and profound suppression of T cell activation (31).

Anticoagulants are commonly used for blood collection activity and heparin has a well-known anticoagulant, and is a member of the family of polysaccharides, the glycosaminoglycans (GAGs) (32). Furthermore, independent of anticoagulant activities many of the anti-inflammatory activities of heparin are now known (33). Heparin has been shown to modify many other aspects of inflammatory processes involving neutrophils. For example, heparin has the

ability to inhibit the binding of IL-8 to neutrophils (34), neutrophil degranulation and the respiratory burst is also inhibited by heparin (35).

Ethylene Diamine TetraAcetic Acid (EDTA) is another anticoagulant currently recommended for routine full blood cell counts (36). The effect of anticoagulants on the isolation of human neutrophils was assessed and have shown that EDTA provided the highest number of isolated neutrophils /ml of blood compared with heparin (37).

In normal peripheral blood mononuclear cells (PBMCs) isolation, neutrophils co-purify in the erythrocyte fraction and not within PBMCs when blood is collected using tube containing of EDTA. However, arginase-expressing CD15+ cells also co-purify in the PBMC fraction, and because of their difference in density, this population is named low-density granulocytes (LDGs). The frequency of LDGs was significantly higher in the PBMCs of active VL patients. After getting treatment, the frequency of LDGs was considerably lower (38). PBMCs from active VL patients are characterized by their inability to elicit an immune response, as shown by impaired proliferation and production of IFN-γ (39,40).

Based on extensive studies done in *L. major* infected inbred strains of mice, healing of disease and host defense against Leishmania infection is mainly depend on the development of a Th-1 type response, characterized by the production of IFN-γ and non-healing is associated with Th-2 type of immune response dominated by the production of IL-4 (8).

A recent publication showed unexpected results; they found that there was production of IFN-γ in patients with active VL upon stimulation with *Leishmania* antigen in the whole blood assay, by using tubes containing heparin to draw venous blood and conclude that all antigens tested showed positive IFN-γ responses in active VL with highest value (41).

Moreover, another study has shown that immune responses against the infection in active VL patients are suppressed and PBMCs don't proliferate and produce IFN-γ in response to *Leishmania* antigen (42).

1.3 Rational of the study

A remarkable effect of heparin on the survival of LDGs has been shown, a dramatic decrease in the frequencies of LDGs was observed when the blood was collected into heparin tubes as compared to EDTA (43). Given that in the studies that showed IFN- γ production by VL patients whole blood assay was used and, blood was collected into heparin anticoagulant, it is possible that the resulting low frequency of LDGs was not sufficient to suppress the production of IFN-γ (44). Thus, the hypothesize was the use of heparin as an anticoagulant in the whole blood assay reduced the survival of LDGs and thereby affect T- cell response.

There is no study previously conducted to assess the effect of anticoagulants on the survival of activated, low density neutrophil (LDGs) in visceral leishmaniasis and how this may affect T cell responses. Therefore, this study intended to generate information about the effect of anticoagulants on the frequency of LDG and the release of IFN-γ and IL-10 before and after treatment. So that for the future *in vitro* experiments, researchers will use the correct anticoagulant to study T-cell responses against *Leishmania* antigen and to avoid ambiguous patient results.

2. Literature review

For quite some time, it has been thought that based on studies done in murine *L. major* models in mouse, there is a shift in immune response and it will be dominated by Th2 immune response, which is characterized by the release of IL-4 and IL-5, favors for parasite survival. This will lead to the impairment of CD4+ Th1-cells to produce INF- γ, which is important for activation of macrophages and DCs that leads to parasite death (8,10).

Conversely other studies have shown that there is no a clear Th2 skewing in human VL. During acute phase of infection, high level of IFN-γ detected in blood serum, and elevated levels of IFNγ mRNA has been found in lymphoid organs like spleen and bone marrow. These observations suggest that the immune system is highly activated and the development of VL is not determined by Th2 skewing but other mechanisms may contribute for pathogenesis of VL (40).

As well, study performed in India assessed the production of IFN-γ in whole blood from active VL patients by using Interferon gamma release assay (IGRA). An increased production of IFN-γ reported when stimulated with Soluble *Leishmania* antigen (SLA) in 80% (28/35) patients with no difference in the mean level of IFN-γ compared to cured VL (45).

In addition to this, unexpected findings were reported in a study that have used whole blood Interferon-γ release assay using a modified technique called modified QuantiFERON, which is a potential marker of *L. donovani* infection and there was production of IFN-γ after stimulation with SLA 85% (11/13) patients with active VL (41).

Furthermore, studies in India have shown significantly higher levels of circulating IFN-γ and IL-10 in VL samples before treatment as compared to post treatment of VL (44). Other studies also showed similar results using serum samples from Sicilian patients with confirmed VL and controls. There was a significant increase in the serum concentration of IFN-γ in patients with active VL, compared to controls and returned to normal after recovery (46).

An epidemiological study of visceral leishmaniasis (VL) conducted in south-west Ethiopia, with a group of 109 subjects including healthy controls were studied for T cell subsets and cytokine profiles, the plasma concentrations of both IFN-γ and IL-10 were significantly higher in patients with active VL, when compared to controls (47).

Over all the above studies have shown that there is an increased production of IFN-γ in whole blood assay after activation with SLA and also in sera or plasma of patients with active VL. This could be the effect of heparin on the survival of LDGs and these cells will not be sufficient to suppress IFN-γ production by T-cell (43).

Furthermore, different studies showed activation of the T cell is depend on the availability of L-arginine. During degranulation, PMNs release arginase and this result depletion of extracellular L-arginine and thus T-cell activation become suppressed and unable to produce IFN-γ (29-31).

Studies done in Gondar, Ethiopia shows contradictory idea from the above studies because they identified the phenotype of arginase-expressing cells among PBMCs as neutrophils (known as LDGs) shows that their frequency was increased in PBMCs of patients before treatment by using EDTA as an anticoagulant. This coincides with reduced levels of L-arginine in the plasma and decreased expression levels of T cell receptor ζ chain (CD3ζ) in T cells, principal signal transduction element of the T cell antigen receptor (TCR), which is important for T cell activation. Significantly lower CD3ζ in CD4+ T cells from patients with active VL before treatment was observed when compared to controls and treated patients which shows a higher CD3 ζ in CD4+ T cells (38,48). The authors hypothesized that this results in T cell suppression and impaired capacity to proliferate and produce IFN-γ (49).

The above discrepancy between studies may came from anticoagulants they used, one used heparin and the other EDTA. Here in the present study shows the impact of EDTA and Heparin on the frequency of LDGs, and activation of T cells in patients with active VL before and after treatment.

2. Objective

2.1 General Objective

- To assess the effect of EDTA and Heparin anticoagulants on the survival of LDGs in PBMCs and the release of interferon-γ and IL-10 in the WBA

2.2 Specific Objective

- To determine the effect of EDTA and heparin on the frequency of LDGs in PBMCs from untreated patients with active VL
- To determine arginase activity in EDTA and heparin following antigen-specific and polyclonal simulation in WBA
- To determine the effect of heparin and EDTA on antigen-specific and polyclonal T cells activation in WBA in VL patients before and after treatment

3. Materials and Methods

3.1 Study Design

A cross-sectional study was used

3.2 Study Setting and period

The study was conducted from December 2014 to May 2015 at University of Gondar Hospital and Gondar is found in Amhara region of North west Ethiopia and is around 740 km far from capital city of Ethiopia which has an average population of 300,000 and it has an average altitude of 2160 above sea level, Gondar University Hospital is found in Gondar town and give different health service for the community and it has different research centers.

3.3 Populations

3.3.1 Target population

All patients suspected for visceral leishmaniasis who attended Gondar University Hospital during the study period

3.3.2 Study population

All VL confirmed patients who visited Gondar University Hospital during the study period.

3.3.3 Inclusion Criteria

All clinically and laboratory confirmed VL patients and Willingness to take part in the study

3.3.4 Exclusion Criteria

- HIV- coinfection

- Age less than 18 years

- TB - coinfection

- Malaria-coinfection

3.4 Sampling technique and Sample size

Convenient sampling technique was used. All consecutive clinically and laboratory confirmed a total of 69 VL patients who came to Gondar University Hospital during the study period and 10 controls were included in the study.

3.5 Study Variables

3.5.1 Dependent variables

- Frequency of LDGs
- Arginase activity
- Production of IFN- γ and IL-10

3.5.2 Independent variables

EDTA, Heparin

3.6 Sample collection and procedures

A total of 12 ml of blood sample was collected in EDTA and heparin tubes at baseline before leishmania treatment and also after treatment. Two ml with SLA, 2ml with PHA and 2ml with nil stimulated samples were collected to determine frequency of LDGs, Arginase activity and production of IFN-γ and IL-10.

3.6.1 Laboratory Evaluation

HIV was screened following the national algorithm with the following tests : KHB (Shanghai Kehua Bio-engineering Co. Ltd), HIV ½ STAT- PAK (Chembio) and Uni-Gold (Trinity Biotech PLC). Haematology analysis (platelet counts, white blood cell counts, hematocrit and haemoglobin) were done with a COULTER AcT diff Hematology Analyzer.

3.6.2 Flow Cytometry

Flow cytometry was done using FACS (Flouresent Activated Cell Sorter) Calibur (BD Biosciences) to assess the frequency (% histogram) of LDGs.

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells according to their size and uses the principle of hydrodynamic focusing for presenting cells to a

laser. cells were differentiated by using forward and sideward scatter and monoclonal antibodies labeled with florochrome were bind with the specific cell surface marker.

Antibodies were used as follows: anti-CD15 (Clone H198, BD Pharmingen) labeled with Phycoerythrin (PE) florochrome and Anti-arginase I (HyCult Biotechnology: clone 6G3) labeled with Fluorescein Isothiocyanate (FITC) florchrome. Cells were washed with PBS, the fixation step was performed with 2% formaldehyde in PBS and the permeabilization step (intracellular staining for Arginase) was with 0.5% saponin in PBS and data were analyzed using Summit v4.3 software.

3.6.3 Determination of arginase activity

To activate arginase, 50µl of PBMCs (resuspended in lysis buffer = 0.1% Triton X-100/10 mM $MnCl_2$/ 25 mM Tris-HCl) were incubated for 7 min at 56^0C. Arginine hydrolysis was achieved by incubating the lysate with 50 µl of 0.5 M L-arginine (pH 9.7) at 37^0C for 120 min. The reaction was stopped with 400µl of H_2SO4 (96%)/H_3PO4 (85%)/H_2O (1/ 3/7, v/v/v). Urea concentration was measured at 540 nm after addition of 20 µl a-isonitrosopropiophenone (dissolved in 100% ethanol), followed by heating at 100^0C for 45 min. To determine arginase activity in the plasma (supernatant from stimulated cells), urea concentrations were first determined in the plasma, without the activation and hydrolysis steps; these values were subtracted from those obtained by measuring the urea levels as described above. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 mmol of urea per min.

3.6.4 Antigens (SLA)

Soluble Leishmania antigen (SLA) was prepared from stationary-phase *Leishmania donovani* promastigotes, harvested after the third or fourth passage. Parasites were washed in 15mM phosphate-buffered saline (PBS), pH 7.4, and the pellet ressuspended at a concentration of1×10^8 cells/ml in 15mM PBS, pH 7.4. The suspension was rapidly frozen at 70^oC and thawed at 37^oC, three times and sonicated between each step. After sonication, the lysate was centrifuged (37000g) for 90 min, the supernatant collected, dialyzed overnight against 15mM PBS, pH 7.4, sterilized by filtration through 0.22-mm-pore-size membrane (Filter millex, Milipore Products Division, Bedford, MA, USA) and stored at 70^oC until use.

Phytohaemagglutinin (PHA) and Phosphate buffered saline (PBS) were used as positive and negative controls, respectively.

3.6.5 Collection of blood and stimulation of cells

Two ml of blood was collected in EDTA and 2ml in Heparin containing tubes. Blood collected in the presence of the different anticoagulants was tested for their ability to respond to antigenic stimulation using 5µg/mL of the SLA antigen described above and in each test 10 µg/mL PHA was used as a positive control and unstimulated cells (nil, PBS) were used as negative controls After adding antigens (75µl of SLA for 2ml of blood) and mitogens (20µl of PHA for 2 ml of blood) and PBS (75µl) to the not stimulated group, all tubes were thoroughly mixed. All atubes were incubated upright in a 37^0C incubator for 24 hrs without humidity and CO_2. After incubation and centrifugation at 10,000rpm for 5 min, supernatant plasma were collected and stored at -20^oC for IFN-γ and IL-10 analysis.

3.6.6 Measurement of Interferon-gamma and Interleukin -10 by ELISA

The level of IFN-γ and IL-10 secreted by cells stimulated in the WBA was measured using Human IFN-γ and IL-10 ELISA Ready-SET-Go! kit using manufacturer's instructions. A standard curve with 8 serial dilutions of known amounts of recombinant human IFN-γ (in picograms per milliliter (pg/ml)) was done in each test and used to determine the levels of IFN-γ produced in response to specific stimulation with *Leishmania* antigens, in response to mitogens and to determine the spontaneous release in the nonstimulated control group. Microtiterwells without any test sample but all contents of the ELISA were used to determine the background of the ELISA assay. The IL-10 content of all samples was also tested by ELISA following the same protocol as described for IFN-γ. Antigen-specific IFN-γ and IL-10 levels (expressed in pg/mL) produced in response to SLA stimulation were determined by subtracting background levels measured in the unstimulated nil (PBS) samples. The mitogen-induced IFN-γ and IL-10 levels (expressed in pg/mL) produced in response to PHA stimulation were determined by subtracting background levels measured in the unstimulated nil (PBS) samples.

The detection limit for IFN-γ was 4 pg/ml with the standard curve ranging from 4-500pg/ml (considered as positive IFN γ production), the low detection limit was < 4pg/ml and the upper detection limit and > 500pg/ml. If a test sample was above the high detection limit, the sample

was diluted and the assay repeated to obtain values that fit into the detection limits of the assay and the for the calculation of the cytokine levels in the diluted samples, values measured were multiplied by the dilution factor.

The detection limit for IL-10 was 2 pg/ml and the standard curve ranged from 2-300 pg/ml (considered as positive IL-10 production), the low detection limit was < 2 pg/ml and > 300pg/ml was the high detection limit..

3.6.7 Data Processing and Analysis

Data were evaluated for statistical differences using a two-tailed Mann-Whitney test or Wilcoxon matched-pairs signed ranked test (GraphPad Prism 6) and differences were considered statistically significant at $p < 0.05$. Unless otherwise specified, results are expressed as mean ± standard error of the mean. FACS data were analyzed using Summit v4.3 software.

3.6.8 Ethical considerations

Ethical clearance was obtained from University of Gondar, Institutional Review Board (IRB). Additionally, Informed written consent was obtained from each patients and controls. To ensure confidentiality of participants, only codes were used and their names were not indicated.

3.6.9 Data quality control measures

Data collectors and supervisor trained on the details of the principle and procedure of the test and also laboratory determination was based on Standard Operating Procedures (SOP). Instrument calibration like FACS caliber was calibrated using standard beads, ELISA known positive and negative controls was used.

3.6.10 Dissemination of plan

The results of the study was presented to University of Gondar as a partial fulfillment for the master course in Immunology. Findings will also be presented in national and international scientific conferences and published in international journal.

4. Results

For this study, 23 patients with visceral leishmaniasis, 16 test of cure (TOC), 20 and 10 for 3rd month and 6th month follow up respectively, a total of 69 clinically and laboratory confirmed leishmania patients were enrolled in this study. For purposes of comparison 10 apparently male healthy controls were recruited. The median age of the patients with leishmania infection and healthy controls were 25 and 26.5 respectively.

All patients recruited in the study were migrant workers and male. The diagnosis of VL was based on positive serology (rK39, DiaMed IT Leish, DiaMed AG, Cressiers/Morat, Switzerland) and presence of amastigotes in spleen or bone marrow aspirates. All patients were HIV negative. Most of VL patients were treated with 20 mg/kg/day of SSG + 15 mg/kg/day of paramomysin (PM) combination for 17 days based on their clinical finding and laboratory test and Some of them treated with 5 mg/kg/day of Ambisom for 30 days.

4.1 Impact of EDTA and heparin anticoagulants on the frequency of LDGs in the PBMCs of VL patients

The impact of EDTA and heparin anticoagulants on the frequency of LDGs was assessed. The results show that the frequency of LDGs is significantly lower in the PBMCs isolated from blood collected with heparin(1.80 ± 0.77) as compared to EDTA(5.10 ± 1.91), $p < 0.0001$(Figure 1)

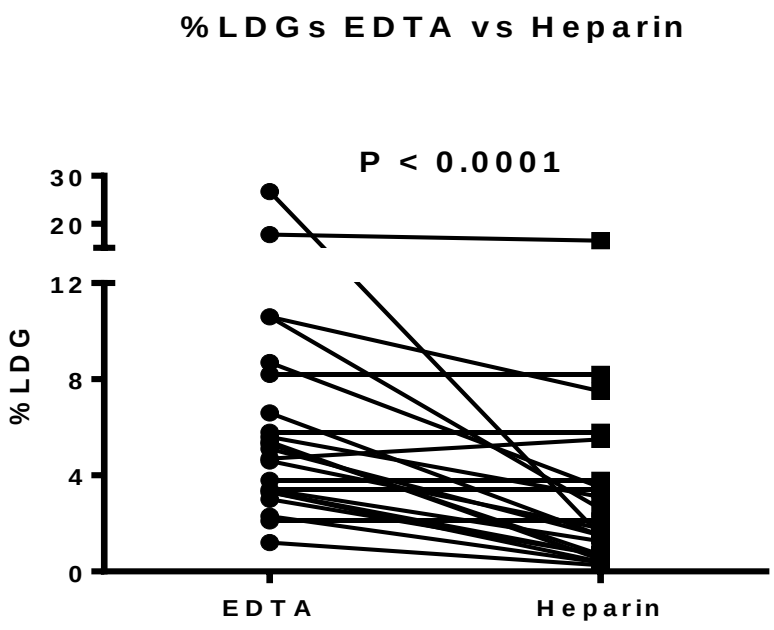
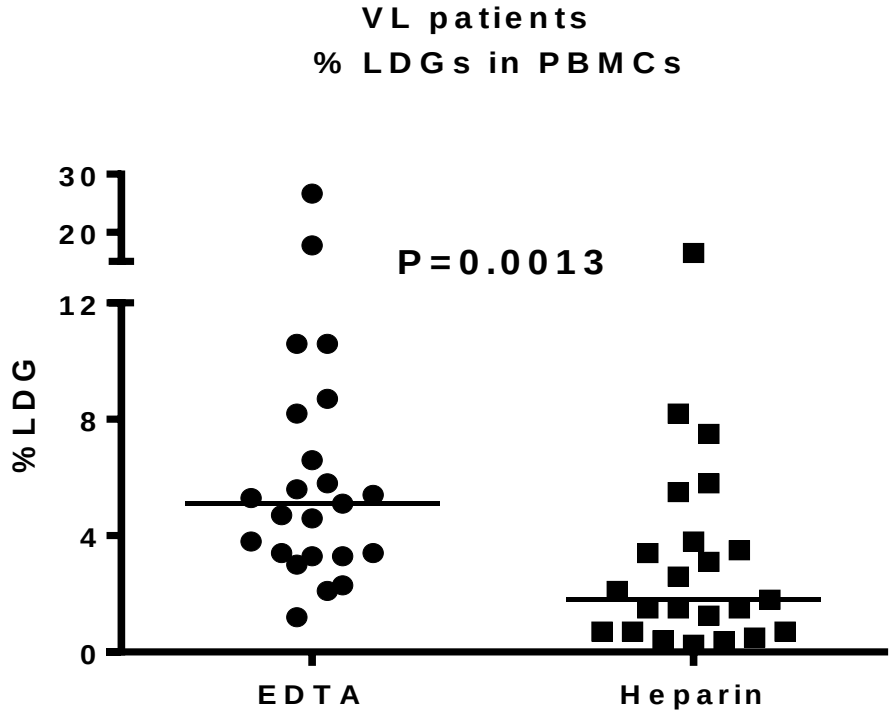


Figure 1: Percentage of low density granulocytes in tubes containing EDTA and heparin. PBMCs were isolated by Ficoll gradient from the blood of VL patients (n = 15). Statistical significance was determined by Wilcoxon paired test.

4.2 Comparison of the impact of different anticoagulants on the frequency of LDGs in the PBMCs of VL patients and controls

The effect anticoagulants on the frequency of LDGs in healthy controls and VL patients was assessed. The results show that during active VL the frequency of LDGs was increased (5.10 ± 1.91) as compared to healthy controls (2.47±0.72), p=0.0013 (Figure 2a,b). In addition, in controls the frequency of LDGs was higher in blood samples collected in EDTA tube than samples collected in heparin tubes (2.47±0.72 Vs 0.4±0.16, p=0.0020), respectively (Figures 2b, 2c). Therefore suggesting that heparin affects the frequency of LDGs not only in active VL but also in controls.

a)



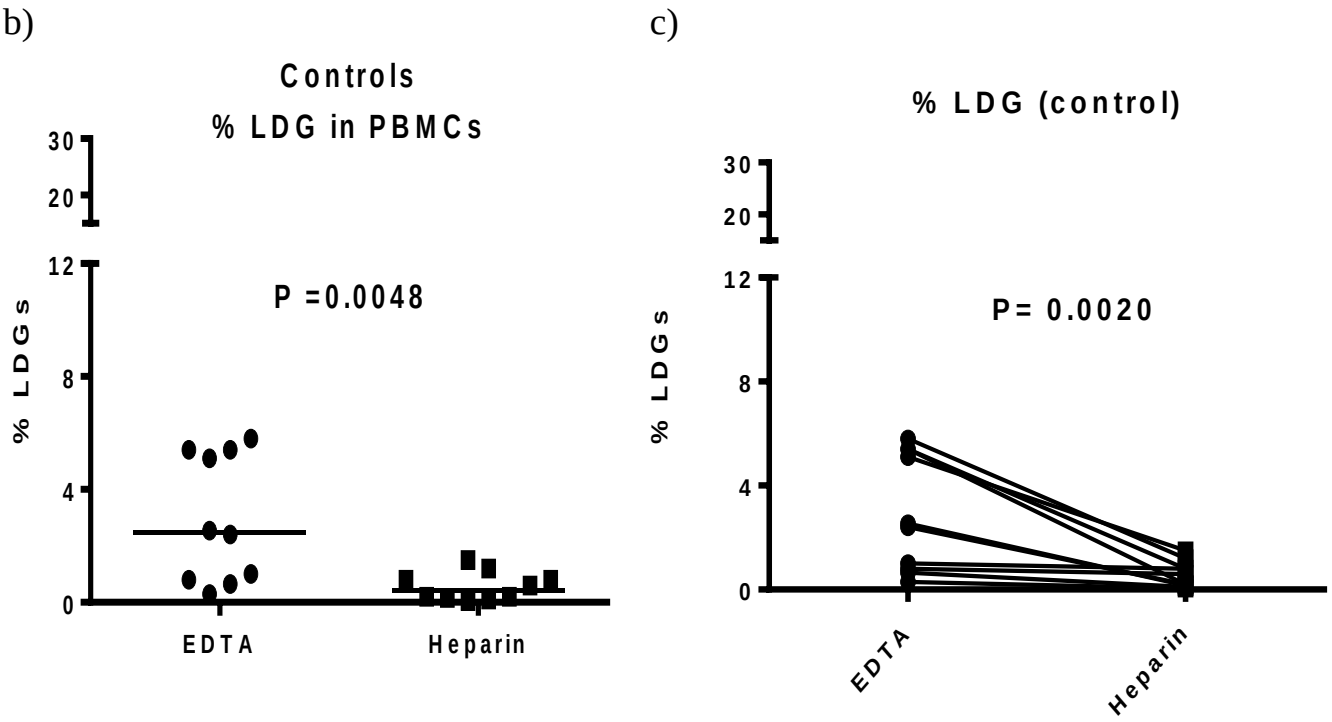


Figure 2 :- Percentages of low density granulocytes in VL patients and controls. PBMCs were isolated by density gradient from the blood of VL patients (n = 23) and controls (n = 10). Each symbol represents the value for an individual. The bars represent the median values for the groups and statistical significance was determined by Mann-Whitney (Figures 2a and b) and Wilcoxon paired tests (Figure 2c).

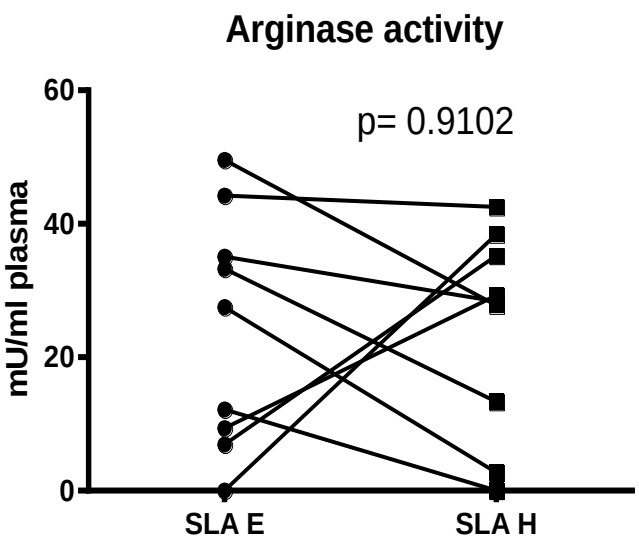
4.3 The effect of EDTA and Heparin on Arginase Activity

The results presented in Figures 1 and 2 show that the frequency of LDGs is lower when blood is collected with tubes containing of heparin anticoagulant compared to EDTA. The effect of using EDTA or heparin as anticoagulant in the release of arginase in the microenvironment by neutrophil was assessed since arginase is released by the azurophilic granules in neutrophils. Accordingly, arginase activity was measured in the supernatant of stimulated cells. It has been found out that the levels of arginase activity difference in the whole blood assay stimulated with specific leishmania antigen (SLA), polyclonal stimulation with PHA and unstimulated (nil) samples (figure 3) did not show any difference between blood samples collected using tubes containing of heparin or EDTA as anticoagulant. Suggesting that the levels of arginase activity were not significantly affected by the different anticoagulant used (Table 1).

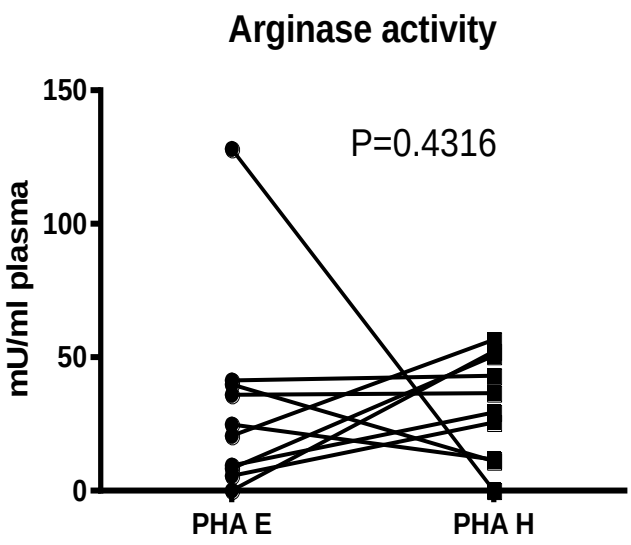
Table 1:- Arginase Activity in the supernatant of stimulated cells with P- value

	median±SEM (Heparin)	median±SEM (EDTA)	P value
SLA	28.10±5.171	19.85±5.807	0.9102
PHA	32.90±6.165	22.65±11.72	0.4316
NIL	24.20±7.932	26.90±5.246	0.8457

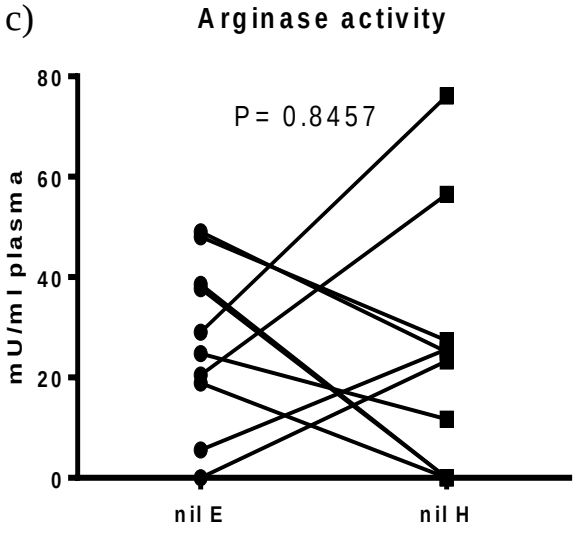
a)



b)



c)



d)

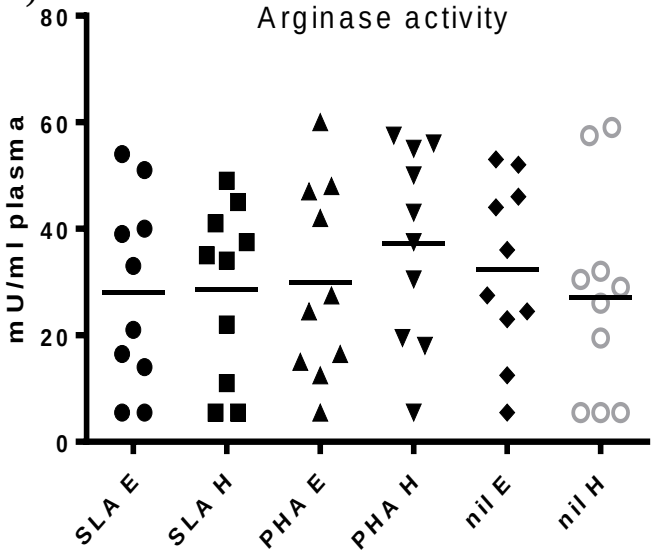


Figure 3 :- Arginase activity in plasma (supernatant). Blood from VL patients (n=10) collected with heparin (H=10) and EDTA (E=10) was activated with SLA, PHA and nil. After 24 hours, the supernatant was harvest and arginase activity was measured by enzymatc assay. Statistical significance was determined by Wilcoxon paired test.

4.4 Impact of anticoagulants on IFN-γ production in the whole blood assay

The effect of use of EDTA and heparin anticoagulants on the levels of antigen-specific and polyclonal production of IFN-γ in the WBA was assessed.

No statistical difference was observed in the level of IFN-γ production between blood samples collected in EDTA and heparin tubes following stimulation with specific antigen for leishmania (0.0 ± 4.9 Vs. 0.0 ± 16.3, p=0.3125 respectively) (figure 4a) and PHA (5.0 ± 18.54 Vs. 0.0±7.4, p=0.6523, respectively)(figure 4b). Shows that IFN-γ production was low or below detection limit in the WBA and that this is unlikely to be due to arginase-induced L-arginine depletion.

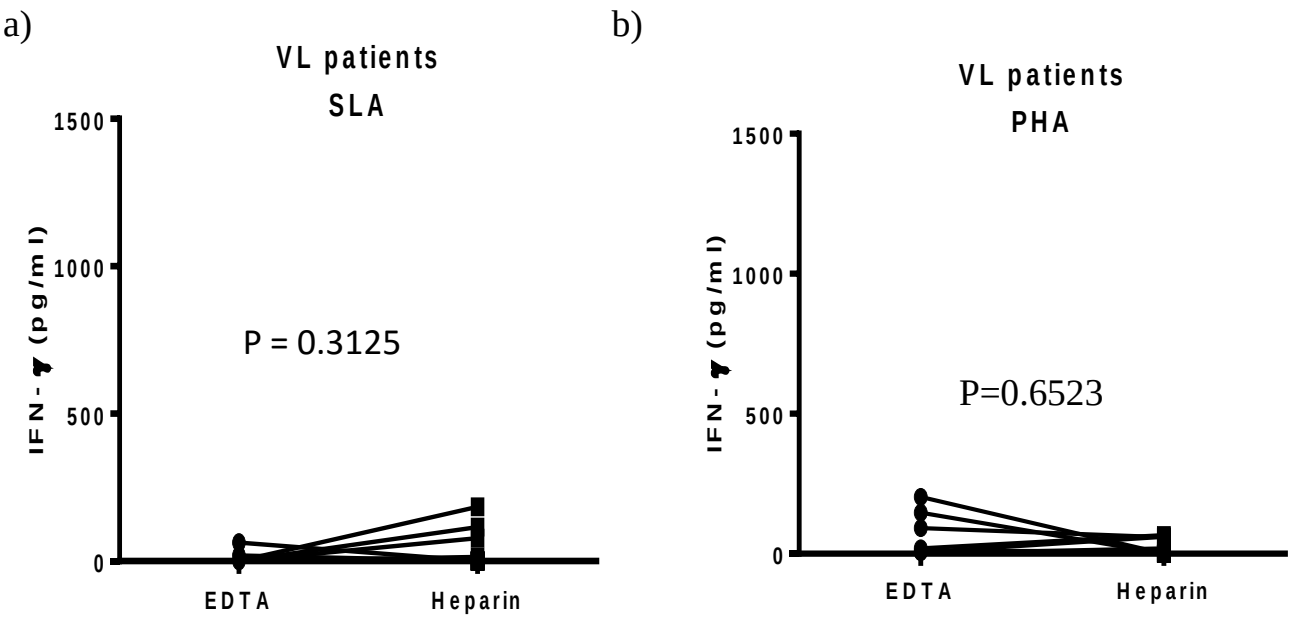


Figure 4:- IFN-γ levels in the supernatant of whole blood assay. (A) Comparison of SLA-stimulated IFN-γ release in pg/ml (unstimulated value subtracted) for VL patients with EDTA and heparin. (B) Comparison of PHA-stimulated IFN-γ release in pg/ml (unstimulated value subtracted) for VL patients with EDTA and heparin. Each symbol represents the value for one individual. Statistical significance was determined by Wilcoxon paired test.

To exclude any technical problem with the assay, the same analysis was done on samples collected from controls. IFN-γ was clearly detectable and Statistical difference was found in response to polyclonal activation, when the blood was collected with heparin, but not EDTA (630.4±166.1, 13.3±20.0, *p*=0.0020, Figure 5a). As expected, IFN-γ was low or below detection limit in the supernatant of whole blood from controls activated with SLA (2.10 ±1.19 pg/ml, data not illustrated).

Results (Figure 5b) show that collecting blood on EDTA prevents the production of IFN-γ, p=0.7969.

a)

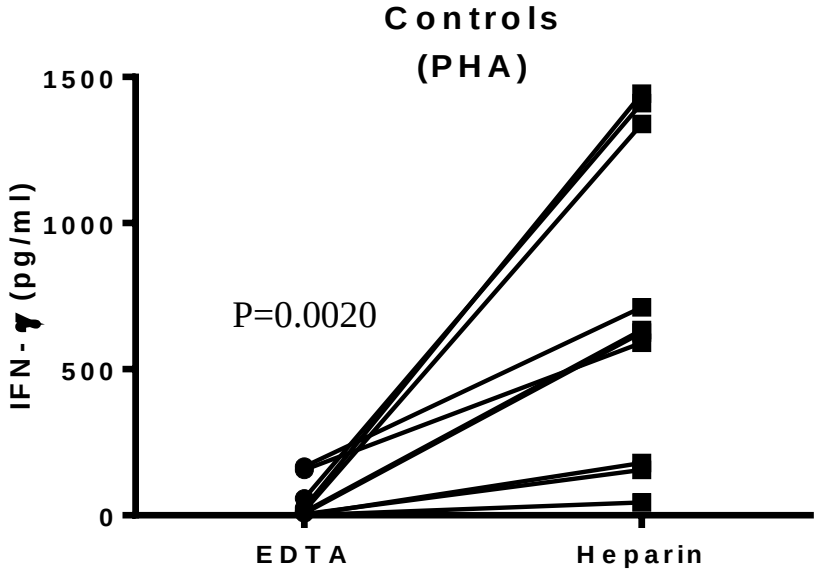


Figure 5 :- IFN-γ levels in the supernatant after stimulation of PHA in whole-blood assay. Comparison of PHA-stimulated IFN-γ release (unstimulated value subtracted) for controls in EDTA and heparin. Statistical significance was determined by Wilcoxen paired test.

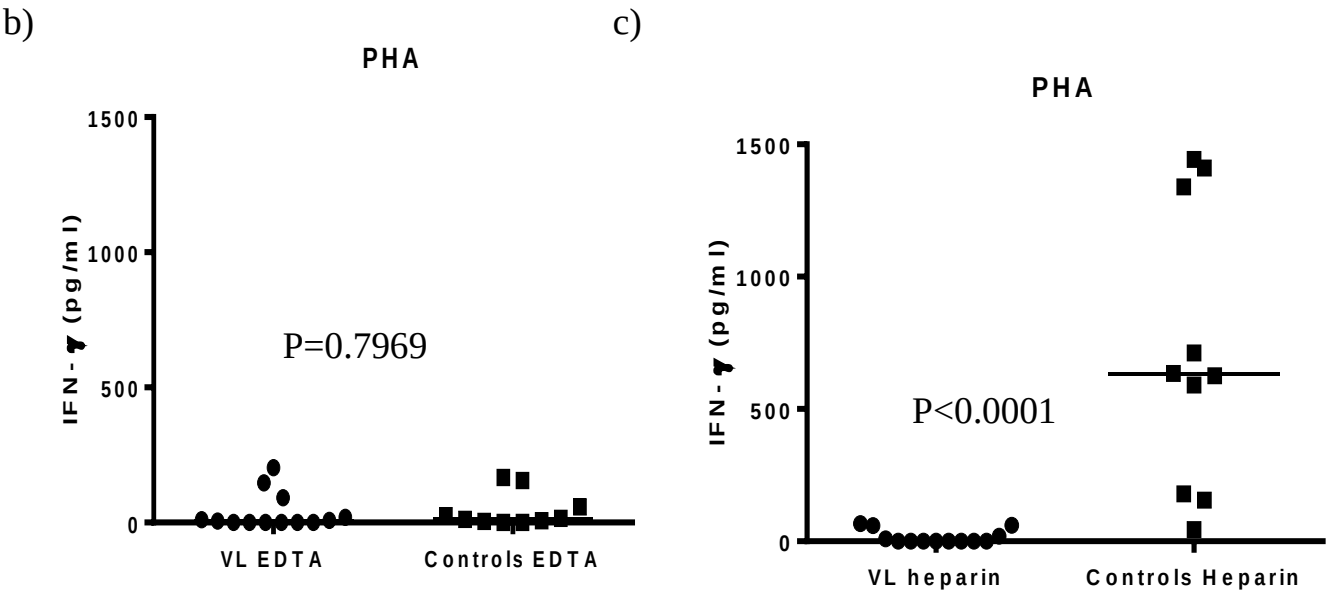


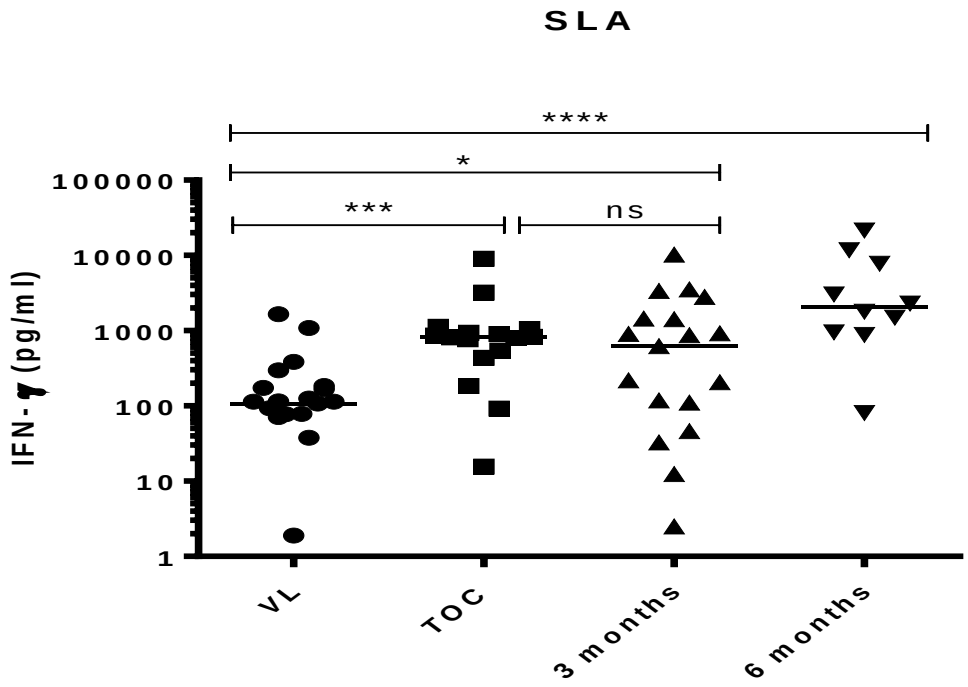
Figure 5 :- IFN-γ levels in plasma after stimulation of PHA in whole-blood assay. Comparison of PHA-stimulated IFN-γ release (unstimulated value subtracted) for VL and controls in EDTA and heparin. Each dot represents the value for an individual. The bars represent the median values for the groups. Statistical significance was determined by Mann-Whitney test.

4.5 Production of IFN-γ in active VL and after treatment

The level of IFN-γ production after successful treatment on test of cure (TOC), 3rd month and 6th month follow up patients were assessed. The results show that the production of IFN-γ with stimulation of SLA on TOC (824.6±541.1), 3rd month (624.7±557.3) and 6th month (2040±2145)(Figure 6a) were increased significantly and gradually after successful treatment.

Similar results was found on stimulation of PHA on TOC (139.8±164.4), 3rd month (798.1±215.2) and 6th month (996.9±943.1) (Figure 6b).

a)



b)

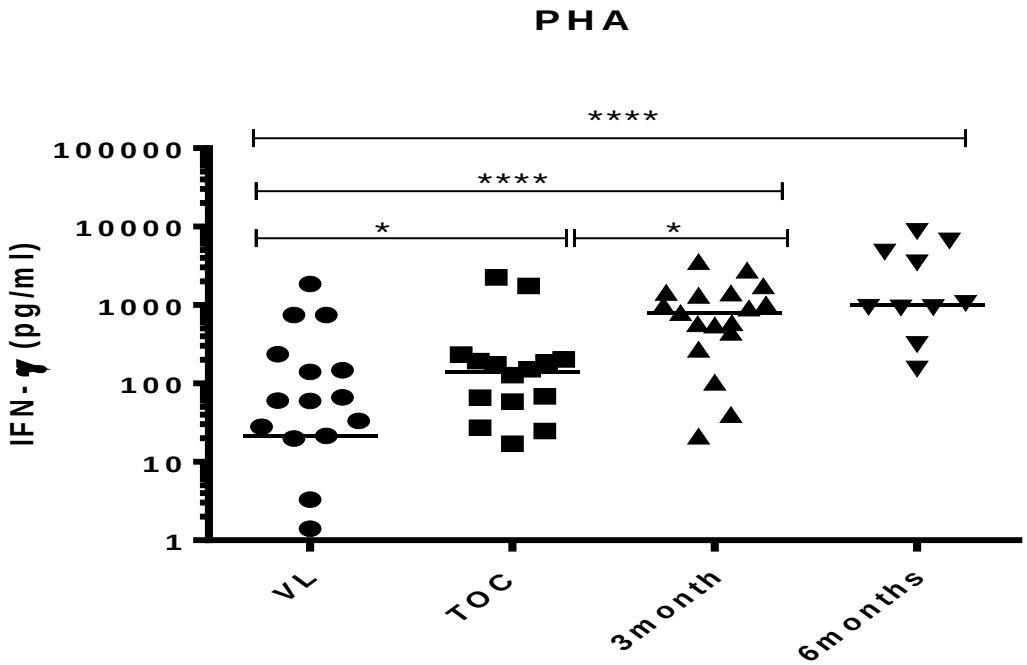


Figure 6:- IFN-γ levels in the supernatant of whole blood assay. (a) Comparison of SLA-stimulated IFN-γ release (unstimulated value subtracted) for different subject groups (b) Comparison of PHA -stimulated IFN-γ release (unstimulated value subtracted) for different subject groups. Each symbol represents the value for an individual. The bars represent the median values for the groups. Statistically significantly different median values for the groups

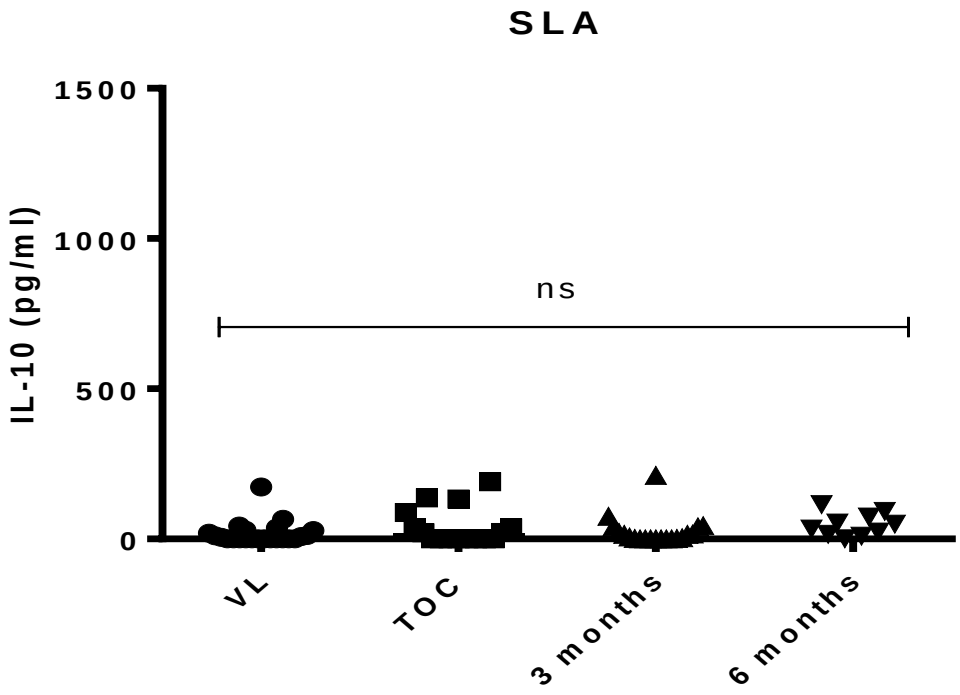
are indicated by bars and asterisks (*, p=0.0102; ***, p=0.0001,****, p < 0.0001; ns, not significant).

4.6 IL-10 production in active VL and after treatment

The production of IL-10 in the supernatant of whole blood samples stimulated with SLA were assessed. No statistical difference was observed in the level of IL-10 production in the supernatant of whole blood samples stimulated with SLA between active VL, TOC (10.35±15.32), 3rd month (10.90±11.96) and 6th month (41.15±11.82) follow up (figure 7a). Suggesting that, antigen-specific IL-10 levels were low or below detection limit during active VL and after successful treatment.

The same analysis was done in the supernatant of whole blood samples stimulated with PHA. statistical difference was observed between active VL, TOC (136.4±46.59), 3rd month (215.1±45.75) and 6th month (749.2±106.8) follow up, p<0.0001 (figure 7b). shows gradually increasing levels of IL-10 after successful treatment

a)



b)

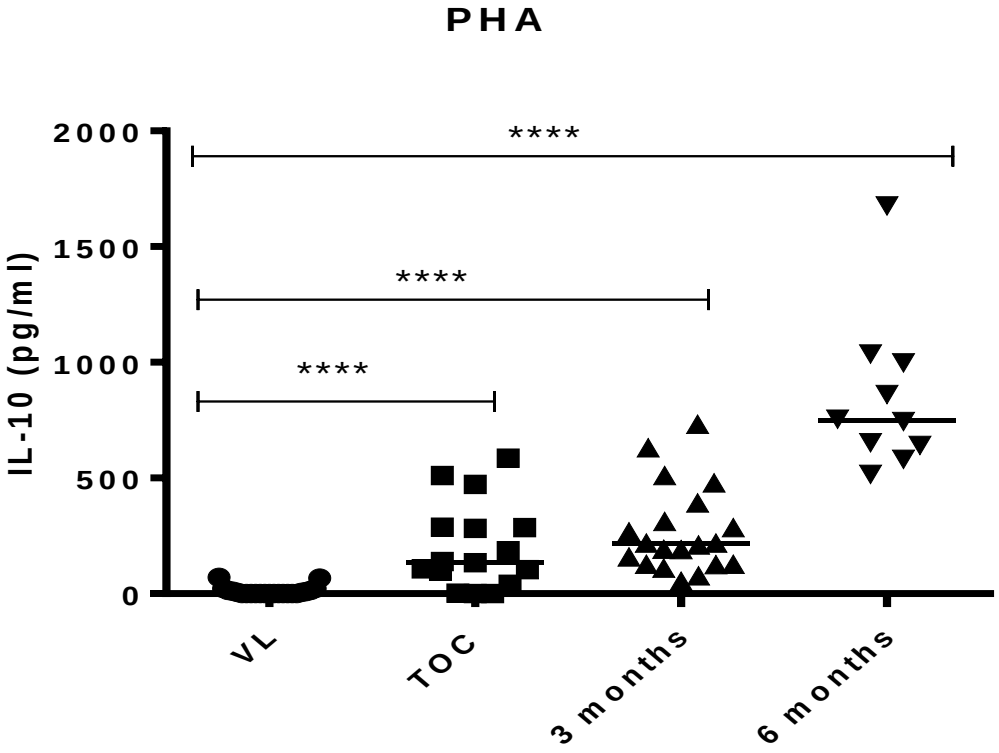


Figure 7:- IL-10 levels in plasma after stimulation with SLA and PHA in whole-blood assay. (a) Comparison of SLA-stimulated IL-10 release (unstimulated value subtracted) for different subject groups (b) Comparison of PHA -stimulated IL-10 release (unstimulated value subtracted) for different subject groups. Each symbol represents the value for an individual. The bars represent the median values for the groups. Statistically significantly different median values for the groups are indicated by bars and asterisks (****, $p < 0.0001$; ns, not significant).

5. Discussion

Recently, the whole blood assay challenged the existing dogma on T cell hyporesponsivness during active VL by showing that VL patients from India maintain the capacity to produce IFN-γ; these studies suggest that the inability of these patients to control the disease was not due to a defect in Th1 response. Indeed, the preliminary data suggested that the use of heparin, the anticoagulant used in the WBA discussed above, results in a sharp reduction on the frequency of LDGs.

Results show that the frequency of LDGs is drastically reduced when the blood is collected with heparin, suggesting that the use of heparin as anticoagulant could result in underestimated frequencies of LDGs. Whereas EDTA has been shown to impact on neutrophils biological functions, the viability of neutrophils does not seem to be affected by different anticoagulants (37).

EthyleneDiamineTetraAcetic Acid (EDTA) chelates the free calcium needed as cofactors to activate the enzymes responsible for coagulation, whereas heparin blocks coagulation by activating antithrombin (37). LDGs are a distinct subpopulation of highly activated neutrophils and with a very short half-life *in vitro* (unpublished data). It is possible that LDGs isolated from blood harvested on EDTA lacks Ca^{+} required to undergo cell death and therefore survive longer in EDTA.

Release of arginase in the microenvironment results in the depletion of extracellular L-arginine, that in turn prevent T cell activation (50,51). Results have shown that in patients with active VL, the frequency of activated LDGs in PBMCs is significantly increased, that these cells express significantly less intracellular arginase and that the levels of arginase in the plasma is significantly increased (38).

The hypothesis was that the lack of IFN-γ response in the WBA could be due to increased released of arginase: however, the present study shows that the levels of arginase activities are similar in all plasma harvested 24 hours after activation of whole blood cells and therefore is unlikely to explain the hyporesponsiveness of the cells. However, additional results in this study shows that:

Cells from whole blood collected on EDTA produce low or no IFN-γ, this is likely to be due to the calcium chelating by EDTA. since Ca^{2+} ion is important for signaling pathway, cell division, proliferation and differentiation to effector T cell. as a result, this will restrict calcium-dependent activation responses (52,53).

Cells from whole blood collected from patients with active VL produce low or no IFN-γ in response to antigen or polyclonal activations, suggesting that blood cells from active VL patients are hyporesponsive. In this study any technical problems were excluded due to the assay that IFN-γ was clearly detectable the supernatant of whole blood cells from healthy controls activated with PHA.

This results are in agreement with an evidence from the literature showing that one of the key immunological characteristics of active VL is a profound immunosuppression, as demonstrated by the failure of PBMCs to produce IFN-γ and proliferate in response to *Leishmania* antigen (reviewed in (39,40)).

However, the results in this study are in apparent contradiction with recent studies showing that IFN-γ is produced by whole blood cells from active VL patients and was similar to those levels detected in cured patients (41,45,54).

Furthermore, the results in this study also shows high IL-10 is not associated with active VL, nor that it is produced at high levels by cells from cured patients. In addition, these results show that cells from active VL patients are hyporesponsive, as activation with PHA results in significant increased of IL-10 only in cured patients after successful treatment.

Taken together, the cytokine results show that blood cells from VL patients are hyporesponsive to both antigen-specific and polyclonal activation. The discrepancies between the results presented here and the studies by (45) and (55) might be explained by several factors:

The severity of the disease might be more acute in VL patients admitted to Gondar university hospital; clinical data such as BMI, anemia, co-infections, age have not been compared in this respective studies in Bihar and in Gondar.

There might be genetic variation between the parasites. Indeed, there is an increased resistance of *L. donovani* to SSG in India, but not in Ethiopia (56).

On the other hand, patients in India respond successfully to treatment with one single dose AmBisome®, but a similar clinical trial had to be terminated because of low efficacy of the drug in Gondar (57).

Despite the fact that it has been found little or no production of IFN-γ and IL-10 in the plasma of the WBA at time of acute disease, these cytokines have been clearly detected in the plasma of these patients directly *ex vivo* (summarised in (39)). demonstrating that these cytokines are been produced *in vivo*.

Indeed, whereas the levels of these cytokines in the WBA in response to antigenic or polyclonal activation was below or barely above the levels of cytokines detected in the absence of stimulation, IFN-γ and IL-10 were detectable in the supernatant of the unstimulated whole blood cells (185.9±178.7 and 61.5±12.6 ng/ml, respectively) as well as in the plasma of these patients (123.0±27.5 and 88.9±12.5 ng/ml, respectively) (data not illustrated).

This demonstrate that this cytokines are produced *in vivo*, but cannot be induced to be produced *in vitro* in response to activation. It is tempted to speculate that other cells, such as neutrophils and monocytes produce these cytokines (39).

In the current study, results show that whole blood cells from active VL patients are hyporesponsive as no or low IFN-γ was induced in response to activation. Since a recent study showed that IFN-γ produced by antigen-specific $CD4^+$ T cells contributes to the control of parasite replication in VL patients, it is possible that the lack of appropriate Th1 response might be responsible for the uncontrolled replication of parasite in the patients that are recruited in Gondar (54).

6. Conclusion and recommendations

Heparin is a well known anticoagulant and causes a dramatic decrease on the frequency of LDGs. EDTA has an effect on the production of IFN-γ. The levels of arginase activity are not significantly affected by the different anticoagulant used. Active VL patients are hyporesponsive and that high IL-10 is not associated with active VL. As a result, choosing different anticoagulants to study about different cells and T cell response against leishmania is important for the future in vitro experiment and also to avoid ambiguous results.

7. Limitation

Demographic data and clinical finding such as age, anemia, BMI and duration of illness were not compared with the Indian study.

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Annex:

Annex: I Consent Form

Title of the Study:- The effect of EDTA and Heparin anticoagulants on the survival of Neutrophils and T-cell responses among Visceral Leishmaniasis Patients in North West Ethiopia

I am a student at University of Gondar and planning to assess the impact of Heparin and EDTA anticoagulant on the survival of neutrophils and the release of interferon-γ and IL-10 in North West Ethiopia. I believe that this study is important to give a valuable information about the effect of anticoagulant on neutrophil and the release of interferon-γ and IL-10. This would help future in vitro experiment for researchers to use the correct anticoagulant type while studying T-cell response against leishmania. You/your child are confirmed to have this disease and If you wish to participate in this study you will be asked to give 8ml blood sample and I will use some of the information on your hospital chart, There is no additional risk incurred to you due to your participation in this study. Your participation to this study should be fully voluntary and you have the right to withdraw from the study at any time during the study. You will not get any additional benefits or compensations by participating in this study except for transportation re-imbursement. The findings of this study will help to have a good information about the anticoagulant effect. We assure you that any information collected from you for the purpose of the study will be kept confidential at all times.

For participation into the study

I, the undersigned, would like to confirm that, in giving my consent to participate in the study, it is with a clear understanding of the objectives and conditions of the study and with the recognition of my right to resign from the study.

I …….……do hereby give consent to Mr./Ms. _____to include me in the proposed research to test the blood. I have been given the necessary information to understand that it will be necessary to collect the blood and that there are no additional risks involved in donating the samples and participating in this research. I have been assured that I can withdraw my consent at any time without penalty or a loss of benefit. The proposal has been explained to me in the language I am fluent and conversant.

Patient

Print Name:		Date:	
Sign Name:		Time:	

Person Conducting the Informed Consent

Print Name:		Date:	
Sign Name:		Time:	

Witness

Print Name:		Date:	
Sign Name:		Time:	

Contact person:

Name: _____

Address: _____

Consent form Amharic version

የጥናቱ ርዕስ : በካላዛር በሽተኞች ላይ የደም ናሙና በመጠቀመም በላብራቶሪ ውስጥ ደም እንዳይረጋ የሚያደርጉት ንጥረ-ነገሮች በነጭ የደም ተህዋስያን ላይ የሚያደርሱት የጎንዮሽ ጉዳት ላይ የሚደረግ ጥናት በሰሜን ምእራብ ኢትዮጵያ፡፡

እኔ የጎንደር ዩኒቨርሲቲ ተማሪ ስሆን በሰሜን ምእራብ ኢትዩጵያ ዉስጥ በሚገኙ የካላዛር በሽተኞች ላይ ጥናት ማድረግ እፈልጋለሁ፡፡ጥናቴም የሚያተኩረው በላብራቶሪ ውስጥ ደም እንዳይረጋ የሚያደርጉት ንጥረ-ነገሮች ማለትም ሄፓሪን እና ኢዲቲኤ በነጭ የደም ተህዋስያን እና በሽታውን ለመከላከል ከሚለቀቁት ንጥረ-ነገሮች የሚያደርሱት ጉዳት ላይ ነው፡፡እናም ይህ ጥናት ደም እንዳይረጋ በሚያደርጉት ንጥረ-ነገሮች ላይ ጥሩ እውቀት እንዲኖረን ያደርጋል የሚል እምነት አለኝ፡፡ እንዲሁም ለወደፊት ተመራማሪዎች ትክክለኛውን ንጥረ-ነገር በመጠቀም ለበሽታው የሚያደረግ የመከላከል አቅምን ለማወቅ ይረዳል፡፡በመሆኑም እርስዎ/ልጅዎ ላይ ይህ በሽታ እንዳለ ተረጋግጧል፡፡ በዚህ ጥናት ላይ ለመሳተፍ ከፈቀዱ የደም ናሙና እንዲሰጡ ይጠየቃሉ እንዲሁም ከሆስፒታል መዝገብዎ ላይ የተመዘገቡ አንዳንድ መረጃዎችን ለጥናቱ እንጠቀማለን እናም የሰጡትን የደም ናሙና በመጠቀም የምርምር ስራዉን የምናከናዉን ይሆናል፡፡በዚህ ጥናት ላይ በመሳተፍዎ የሚደርስብዎ ምንም አይነት ጉዳት የለም፡፡ በዚህ ጥናት ላይ ሲሳተፉ በሙሉ ፈቃደኝነት መሆን ይኖርበታል፤ እንዲሁም በጥናቱ ላይ ሲሳተፉ በማንኛዉም ሰዓት ከጥናቱ የማቋረጥ መብትዎ ሙሉ በሙሉ የተጠበቀ ነዉ፡፡ በዚህ ጥናት በመሳተፍ የሚያገኙት የተለየ ጥቅማጥቅም አይኖርም፤ ነገር ግን የዚህ ጥናት ዉጤት ለወደፊት ደም እንዳይረጋ በሚያደርጉት ንጥረ-ነገሮች ላይ ጥሩ እውቀት እንዲኖረን ያደርጋል፡፡ ለዚህ ጥናት አገልግሎት የተሰበሰቡ እርስዎን የተመለከቱ መረጃዎች በሙሉ በማንኛዉም ጊዜ በሚስጥር የሚያዙ መሆኑን እናረጋግጥልዎታለን፡፡

ስምምነት ማረጋገጫ

ጥናት ላይ ለመሳተፍ

ስለጥናቱ የተሰጠኝን ማብራሪያ በበቂ ሁኔታ በመረዳት በጥናቱ ላይ ለመሳተፍ ፈቃደኛነቴን እየገለጽኩ፤ በማንኛዉም ሰዓት ከጥናቱ እራሴን የማግለል መብት እንዳለኝም ተረድቻለሁ፡፡
እኔ _____ ከዚህ በታች የፈረምኩት ለአቶ/ወሪት/ወ/ሮ/ _____
በጥናቱ ላይ እንዲያሳትፉኝ እና የደም ናሙና ወስደዉ እንዲመረምሩ ፈቅጃለሁ፡፡

ከጥናቱ የማቋረጥ መብቴ መሆኑን፤ ከጥናቱ ማቋረጤ ከሚደረግልኝ ህክምና የማያስከለክለኝ መሆኑ፤ እንዲሁም ስለጥናቱ አላማ በአፍ መፍቻ ቋንቋየ ተብራርቶልኛል፡፡

ፈቃደኛነት □ሰጠዉ

ስም		**ቀን**	
ፊርማ		**ሰዓት**	

ፈቃደንነታቸዉን የተቀበለዉ

ስም		**ቀን**	
ፊርማ		**ሰዓት**	

የምስክር

ስም		**ቀን**	
ፊርማ		**ሰዓት**	

<u>ጥናቱን የሚያካሂደዉ ሰዉ አድራሻ</u>

ስም: _____

አድራሻ: _____

Annex: II Information Sheet

Case Report Form (CRF)

Demographic Information

1. Code VL -
2. Chart No. _____
3. Sex ☐ Male ☐ Female
4. Age _____Years
5. Height _____ Meter
6. Weight _____ Kilo Gram
7. BMI _____
8. Duration of illness _____
9. Parasitic Load Spleen _____

   Bone Marrow _____
10. Complete Blood Count

- WBC _____
- HGB _____
- HCT _____
- Lymph _____
- RBC _____
- PLT _____
- MCH _____
- MCHC _____
- Granulocyte _____
- Neutrophil _____
- MCV

11. GOT _____

12. GPT _____

13. Spleen Size _____

14. Liver Size _____

15. Upper arm Circumference _____

15. Place of Residence _____

16. Date of Starting Treatment _____

17. End of Treatment _____

Annex: III Laboratory procedures

I. Procedure for PBMCs isolation for EDTA and Heparin

1. Pipette 4ml Ficoll Histopaque 1077 into a 4ml centrifuge tube
2. Slowly pipette 4ml blood on top of Ficoll (slant tube when dispensing blood)
3. Centrifuge at 1900rpm with no brake for 30 minutes at room temp.
4. Using a sterile pipette collect the interphase below the plasma (PBMC) (collecting as little Ficoll as possible)
5. Wash the interphase with PBS (fill centrifuge tube)
6. Centrifuge 1800rpm for 5 minutes at room temp.
7. Pour away the supernatant and dilute the sediment with 2ml of PBS.
8. Use the PBMCs for FACS analysis by adding monoclonal antibody (anti-CD_{15} and anti-Arginase)

II. Procedure for arginase assay

1. Defrost samples
2. Vortex defrosted sample and centrifuge 10 min 2300rpm
3. Label 1.5ml eppendrof tubes
4. Pipette 25µl of samples in all the tubes
5. Pipette 25µl of lysis buffer to each sample
6. Vortex (can leave at 4°C if necessary, vortex before use)
7. Using a thermal cycler, incubate at 56°C for 7 minutes this activates the arginase present in the sample.
8. Take an aliquot of L-arginine (0.5M at pH9.7) from the freezer, thaw
9. Pipette 50ul L-arginine into each tub.
10. Vortex
11. Using the same thermal cycler, incubate at 37°C for 120 minutes.

12. Pipette and add 400ul acid mixture to stop the reaction (wear lab coat, gloves and GOGGLES!) (Can leave at 4C if necessary)

13. In 6 new 0.5ml eppendorfs, pipette London water and urea standard:

Table -4 :- Urea standard preparation.

Tube number	**London Water (µl)**	**Urea Volume (µl)**	**Urea Conc. (µg)**
1	50	50	60
2	75	25	30
3	87.5	12.5	15
4	93.8	6.2	7.5
5	96.9	3.1	3.75
6	98.4	1.6	1.88

14. Pipette and add 400µl acid mixture to the urea standards (GOGGLES!!!!) (Can leave at 4°C if necessary)

15. Pipette 20ul 6% ISPF to ALL sample and standard tubes.

16. VORTEX TUBES EXTREMELY WELL until the phases are mixed – this is EXTREMELY IMPORTANT!!!!

17. Using the same thermal cycler, incubate all samples and standards at 99°C for 45minutes using a weight on top of the tubes.

18. Incubate a further 30minutes at 4^{o}C

19. When the urea reacts with the coloring agent ISPF it produces a pink to purple color.

20. Pipette 200µl of urea standard and the samples onto a 96 flat bottom well plate (non-sterile plate)

21. Read the absorbance of both the standard and the sample at 540nm

Calculation

Arginase Activity

$$\frac{\mu g \text{ urea} \times \text{dilution} \times \text{homogenate volume} \times \text{mU}}{\mu M \text{ urea}(60\mu g) \times \text{time in minutes} \times \text{homogenate fraction volume}} = \text{mU/ml}$$

III. ELISA procedure for IFN-γ and IL-10

1. Coat Corning Costar 9018 (or Nunc Maxisorp®) ELISA plate with 100 µL/well of capture antibody in 1X Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Seal the plate and incubate overnight at 4°C.
2. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer*. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.
3. Dilute 1 part 5X ELISA/ELISPOT Diluent with 4 parts DI water.* Block wells with 200 µL/well of 1X ELISA/ELISPOT Diluent. Incubate at room temperature for 1 hour.
4. Optional: Aspirate and wash at least once with Wash Buffer.
5. Using DI water, reconstitute lyophilized standards as noted on the C of A. Allow to sit for 15 minutes with gentle agitation prior to diluting further
6. Using 1X ELISA/ELISPOT Diluent*, dilute the reconstituted standard as noted on the C of A to prepare the top standard concentration. Add 100 µL/well of top standard concentration to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. Add 100 µL/well of your samples to the appropriate wells. Include at least two wells with 100 µL/well of 1X ELISA/ELISPOT Diluent only to serve as plate blanks. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).
7. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes**.
8. Add 100 µL/well of detection antibody diluted in 1X ELISA/ELISPOT Diluent * (dilute as noted on C of A). Seal the plate and incubate at room temperature for 1 hour.
9. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes**.

10. Add 100 µL/well of Avidin-HRP* diluted in 1X ELISA/ELISPOT Diluent (dilute as noted on C of A). Seal the plate and incubate at room temperature for 30 minutes.

11. Aspirate and wash as in step 2. In this wash step, soak wells in Wash Buffer* for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes**.

12. Add 100 µL/well of 1X TMB Solution to each well. Incubate plate at room temperature for 15 minutes.

13. Add 50 µL of Stop Solution to each well.

14. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyze data.

NOTES: * Be certain that no sodium azide is present in the solutions used in this assay, as this inhibits HRP enzyme activity.

**The number of washes in the protocol was adapted to an automatic plate washer. This can be decreased when using other methods but should be tested empirically. Allowing time for soaking (~1 minute) during each wash step increases the effectiveness of the washes.